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# Synaptotagmins form a hierarchy of exocytotic $Ca^{2+}$ sensors with distinct $Ca^{2+}$ affinities

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**Synaptotagmins constitute a large family of membrane proteins implicated in $Ca^{2+}$-triggered exocytosis. Structurally similar synaptotagmins are differentially localized either to secretory vesicles or to plasma membranes, suggesting distinct functions. Using measurements of the $Ca^{2+}$ affinities of synaptotagmin $C_2$-domains in a complex with phospholipids, we now show that different synaptotagmins exhibit distinct $Ca^{2+}$ affinities, with plasma membrane synaptotagmins binding $Ca^{2+}$ with a 5- to 10-fold higher affinity than vesicular synaptotagmins. To test whether these differences in $Ca^{2+}$ affinities are functionally important, we examined the effects of synaptotagmin $C_2$-domains on $Ca^{2+}$-triggered exocytosis in permeabilized PC12 cells. A precise correlation was observed between the apparent $Ca^{2+}$ affinities of synaptotagmins in the presence of phospholipids and their action in PC12 cell exocytosis. This was extended to PC12 cell exocytosis triggered by $Sr^{2+}$, which was also selectively affected by high-affinity $C_2$-domains of synaptotagmins. Together, our results suggest that $Ca^{2+}$ triggering of exocytosis involves tandem $Ca^{2+}$ sensors provided by distinct plasma membrane and vesicular synaptotagmins. According to this hypothesis, plasma membrane synaptotagmins represent high-affinity $Ca^{2+}$ sensors involved in slow $Ca^{2+}$-dependent exocytosis, whereas vesicular synaptotagmins function as low-affinity $Ca^{2+}$ sensors specialized for fast $Ca^{2+}$-dependent exocytosis.**

*Keywords*: $C_2$-domain/$Ca^{2+}$ binding protein/exocytosis/neurotransmitter release/synaptic plasticity

## Introduction

When an action potential invades a presynaptic nerve terminal, $Ca^{2+}$ influx triggers neurotransmitter release (Katz, 1969). $Ca^{2+}$ activates two components of release: a fast synchronous component that accounts for >90% of total release, and a slow asynchronous component that mediates <10% of total release (Geppert *et al*., 1994; Goda and Stevens, 1994; Atluri and Regehr, 1998). Both components exhibit a high degree of $Ca^{2+}$ cooperativity but different apparent $Ca^{2+}$ affinities (Dodge and Rahamimoff, 1967; Goda and Stevens, 1994). In addition to stimulating release, $Ca^{2+}$ modulates release during short-term synaptic plasticity (Katz and Miledi, 1968; Dittman *et al*., 2000). Short-term synaptic plasticity is at least partly induced by accumulation of residual $Ca^{2+}$ during repetitive stimulation (Delaney and Tank, 1994; Kamiya and Zucker, 1994) and is an important determinant of the properties of neuronal networks (Dobrunz and Stevens, 1999; Gil *et al*., 1999).

$Ca^{2+}$ is thought to trigger release and regulate synaptic plasticity by binding to $Ca^{2+}$ sensors. Although many $Ca^{2+}$ binding proteins have been identified that could potentially serve as synaptic $Ca^{2+}$ sensors, the best current candidates at the synapse are synaptotagmins 1 and 2, abundant synaptic vesicle proteins that bind $Ca^{2+}$. Synaptotagmins 1 and 2 are highly homologous but differentially distributed, with synaptotagmin 1 primarily expressed in forebrain and synaptotagmin 2 in the brain stem and spinal cord (Ullrich *et al*., 1994; Marqueze *et al*., 1995). However, at least 11 other synaptotagmins are present in brain besides synaptotagmins 1 and 2. Of these 'other' synaptotagmins, synaptotagmins 3 and 7 are the most abundant (reviewed in Südhof, 2002). Different from synaptotagmins 1 and 2, synaptotagmins 3 and 7 are uniformly co-distributed throughout the brain (Ullrich and Südhof, 1995) and are localized to plasma membranes instead of to synaptic vesicles (Butz *et al*., 1999; Sugita *et al*., 2001).

All synaptotagmins are composed of an N-terminal transmembrane region, a linker sequence and two C-terminal $C_2$-domains (referred to as the $C_2A$- and $C_2B$-domains). In synaptotagmin 1, the $C_2A$-domain binds three $Ca^{2+}$ ions, while the $C_2B$-domain binds only two $Ca^{2+}$ ions (Südhof and Rizo, 1996; Ubach *et al*., 1998; Fernandez *et al*., 2001). Both $C_2$-domains bind $Ca^{2+}$ ions with a low intrinsic affinity (>500 μM $Ca^{2+}$). The apparent $Ca^{2+}$ affinity of the synaptotagmin 1 $C_2$-domains is dramatically increased in the presence of phospholipid membranes (~5–50 μM $Ca^{2+}$ depending on the lipid composition), presumably because the phospholipid headgroups provide additional coordination sites for the bound $Ca^{2+}$ ions (Zhang *et al*., 1998; Fernandez *et al*., 2001; Fernandez-Chacon *et al*., 2001). In the ternary $C_2$-domain–$Ca^{2+}$–phospholipid complex, the $C_2A$-domain probably contains three $Ca^{2+}$ ions, but the $C_2B$-domain contains only two $Ca^{2+}$ ions. As a result, the $C_2B$-domain complex is more labile, and $Ca^{2+}$-dependent phospholipid binding to the synaptotagmin 1 $C_2B$-domain was only detected recently by methods in which binding was studied in solution (Fernandez *et al*., 2001). The sequences of most $C_2$-domains of other synaptotagmins are very similar to those of synaptotagmin 1, suggesting that most synaptotagmins also form $Ca^{2+}$-dependent phospholipid complexes via both $C_2$-domains (Li *et al*., 1995a,b). $Ca^{2+}$-dependent phospholipid interactions probably con-

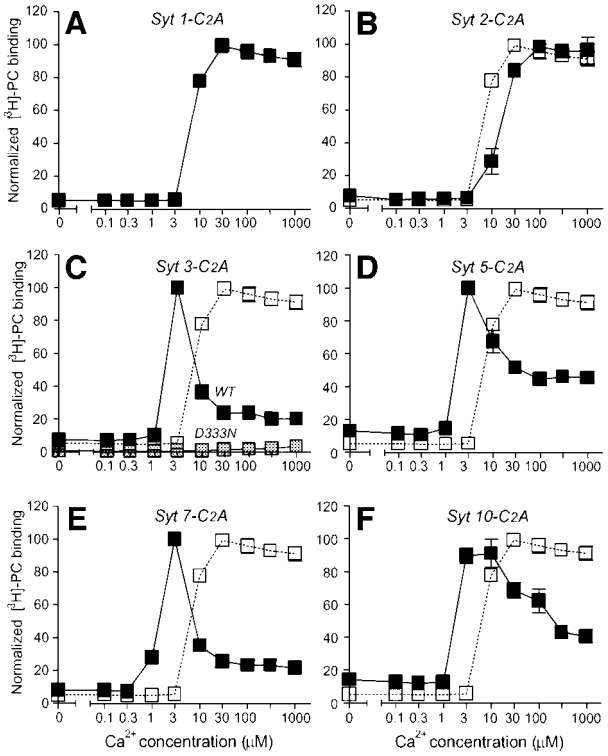


**Fig. 1.** Phospholipid binding to the $C_2$A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10 studied by $Ca^{2+}$-dependent GST pull-downs of radiolabeled liposomes. GST fusion proteins containing the indicated $C_2$A-domains (Syt, synaptotagmin) were immobilized on glutathione–agarose and incubated at increasing concentrations of free $Ca^{2+}$ with radiolabeled liposomes composed of 25% PS/75% PC. $Ca^{2+}$ concentrations were clamped by $Ca^{2+}$/EGTA buffers using the standard NaCl-based buffer (Gerber *et al*., 2001). Agarose beads were washed three times in the respective incubation buffers and bound liposomes were quantified by liquid scintillation counting. Binding was normalized to 100% for the maximal point. Data shown are means ± SEMs from two experiments performed in triplicate. The binding curve for the synaptotagmin 1 $C_2$A-domain in (**A**) is repeated in open squares (**B**–**F**) as an internal reference point to facilitate comparisons. (C) Gray symbols display binding observed for the synaptotagmin 3 $C_2$A-domain (Syt 3-$C_2$A) containing a point mutation in a $Ca^{2+}$ binding loop (D333N).

stitute an intrinsic component of synaptotagmin function since phospholipid binding is the only confirmed function of $C_2$-domains, as exemplified by enzymes such as phospholipase A2 or protein kinase C in which the $C_2$-domain mediates the $Ca^{2+}$-dependent recruitment of the enzymes to the membrane (reviewed in Nalefski and Falke, 1996; Newton and Johnson, 1998).

Knockout mice revealed that, in hippocampal synapses, synaptotagmin 1 is essential for fast but not for slow $Ca^{2+}$-stimulated neurotransmitter release, suggesting that synaptotagmin 1 is an essential $Ca^{2+}$ sensor for fast exocytosis (Geppert *et al*., 1994). This hypothesis is supported by mutant mice in which a point mutation, R233Q, was introduced into synaptotagmin 1 by homologous recombination (Fernandez-Chacon *et al*., 2001). The R233Q mutation caused an ~2-fold reduction in the overall $Ca^{2+}$ affinity of synaptotagmin 1, and a similar decrease in the apparent $Ca^{2+}$ affinity of transmitter release, suggesting that the $Ca^{2+}$ affinity of synaptotagmin 1 determines the $Ca^{2+}$ affinity of synaptic exocytosis (Fernandez-Chacon *et al*., 2001). In contrast to synapses, however, deletion of synaptotagmin 1 in neuroendocrine cells had a surprisingly small effect on exocytosis. In chromaffin cells, the synaptotagmin 1 knockout caused only a minor decrease in fast exocytosis (Voets *et al*., 2001). Similarly, in PC12 cells lacking synaptotagmins 1 and 2, $Ca^{2+}$ still induced robust secretion (Shoji-Kasai *et al*., 1992). The lack of a requirement for synaptotagmin 1 for most large dense-core vesicle exocytosis, but its necessity for synaptic vesicle exocytosis, suggested that other $Ca^{2+}$ sensors may be more important for large dense-core vesicle exocytosis. Indeed, studies in permeabilized PC12 cells demonstrated that the $C_2$A- and $C_2$B-domains of synaptotagmin 7 potently inhibited exocytosis, whereas the $C_2$A- and $C_2$B-domains of synaptotagmin 1 were without significant effect, indicating that synaptotagmin 7 may constitute a $Ca^{2+}$ sensor for exocytosis in these cells (Sugita *et al*., 2001). However, since synaptotagmin 7 is also present in synapses, this raises the question of why synaptotagmin 7 can substitute for synaptotagmin 1 in PC12 cells but not in synapses.

One possible explanation for this conundrum is that in synapses and endocrine cells, vesicular synaptotagmins may be responsible for fast exocytosis triggered at higher $Ca^{2+}$ concentrations, and plasma membrane synaptotagmins for slower exocytosis stimulated at lower $Ca^{2+}$ concentrations. The more severe phenotype of the synaptotagmin 1 knockout at synapses, and the less severe phenotype in endocrine cells, would then be due to the fact that most synaptic exocytosis, but only a small part of endocrine exocytosis, are mediated by the fast component. However, it is unclear whether plasma membrane and vesicular synaptotagmins have intrinsic functional differences as predicted by this hypothesis, and also whether these differences apply to other synaptotagmins, especially synaptotagmin 3, which is co-localized with synaptotagmin 7 on synaptic plasma membranes but whose location in neuroendocrine cells is unclear. In the present study, we set out to address these issues. Our results demonstrate an unsuspected functional specialization of synaptotagmins whereby plasma membrane synaptotagmins exhibit a higher $Ca^{2+}$ affinity than vesicular synaptotagmins, and even vesicular synaptotagmins are heterogeneous with respect to $Ca^{2+}$ affinity. These findings indicate that at central synapses, a series of $Ca^{2+}$ sensors with distinct affinities may operate in triggering fast release. In large dense-core vesicle exocytosis, by contrast, fusion is probably largely driven by high-affinity synaptotagmins that operate more slowly but require lower $Ca^{2+}$ levels.

## Results

### *Relative $Ca^{2+}$ affinities of the synaptotagmin $C_2$A-domains*

We compared, in the same experiment, the apparent $Ca^{2+}$ affinities of the $C_2$A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10. These synaptotagmins were chosen because synaptotagmins 3 and 7 are the most abundant synaptotagmins after 1 and 2 (Butz *et al*., 1999; Sugita *et al*., 2001), and because synaptotagmins 3, 5, 6 and 10 form a class of closely related synaptotagmins (Fukuda *et al*.,

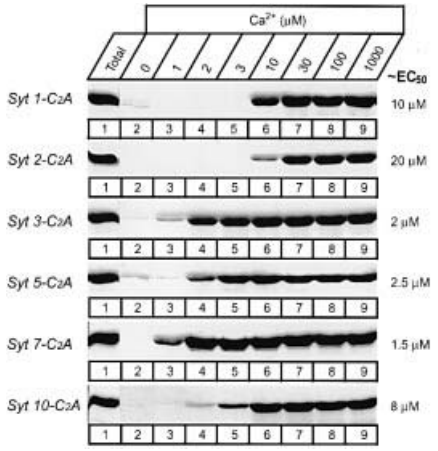


**Fig. 2.** Phospholipid binding by the $C_2$A-domains of synaptotagmins 1, 2, 3, 5, 7 and 10 studied by co-sedimentation with liposomes. Soluble purified GST fusion proteins containing the indicated $C_2$A-domains were incubated with liposomes composed of 25% PS/75% PC in the presence of free $Ca^{2+}$ at the concentrations shown, clamped by $Ca^{2+}$/EGTA buffers. Liposomes were centrifuged and washed, and bound proteins were estimated by SDS–PAGE. Data shown are Coomassie Blue-stained gels from a single representative experiment repeated multiple times. Numbers on the right indicate approximate $Ca^{2+}$ concentrations required for half-maximal binding as estimated from multiple experiments.

1999). Synaptotagmin 6 was not studied because we were unable to produce soluble properly folded $C_2$-domains from this isoform. We estimated the apparent $Ca^{2+}$ affinities of the $C_2$A-domains by $Ca^{2+}$-dependent phospholipid binding, and compared two independent methods and three different buffers to control for potential artifacts. $Ca^{2+}$-dependent phospholipid binding assays were chosen because synaptotagmins bind $Ca^{2+}$ at physiological concentrations only in the presence of phospholipids and because $Ca^{2+}$-dependent phospholipid binding most likely constitutes part of their physiological function (reviewed in Südhof, 2002).

We first measured the apparent $Ca^{2+}$ affinity of the $C_2$A-domains with a standard resin-based assay in which immobilized glutathione *S*-transferase (GST) fusion proteins of the synaptotagmin $C_2$A-domains were incubated with radiolabeled liposomes at different $Ca^{2+}$ concentrations (Davletov and Südhof, 1993). Figure 1 demonstrates that each synaptotagmin is characterized by a distinct $Ca^{2+}$ affinity, as measured by this assay. The vesicular synaptotagmins 1 and 2 consistently had the lowest $Ca^{2+}$ affinities [$EC_{50} \approx$ 10–20 μM $Ca^{2+}$ with liposomes composed of 25% phosphatidylserine (PS)/75% phosphatidylcholine (PC); Figure 1A and B]. By contrast, the plasma membrane synaptotagmins 3 and 7 exhibited the highest $Ca^{2+}$ affinities ($EC_{50} \approx$ 1–2 μM $Ca^{2+}$; Figure 1C and E). Synaptotagmins 5 and 10 (whose localizations are unknown, but which are most closely related to the plasma membrane synaptotagmins 3 and 6) also displayed relatively high $Ca^{2+}$ affinities ($EC_{50} \approx$ 3 μM $Ca^{2+}$; Figure 1D and F). Moreover, we observed that synaptotagmins 1 and 2 (which are both localized to synaptic vesicles but differentially distributed in brain; Ullrich *et al*., 1994; Marqueze *et al*., 1995) also differ in $Ca^{2+}$ affinity (Figure 1A and B), with synaptotagmin 2 reproducibly exhibiting an ~2-fold lower $Ca^{2+}$ affinity than synaptotagmin 1. The specificity of the phospholipid binding reaction was confirmed by a point mutant in the predicted $Ca^{2+}$ binding site of the synaptotagmin 3 $C_2$A-domain (D333N), which abolished $Ca^{2+}$-dependent phospholipid binding (Figure 1C).

The distinct $Ca^{2+}$ affinities of synaptotagmins could have important implications for the $Ca^{2+}$-dependent regulation of neurotransmitter release. This is illustrated by the R233Q point mutation in synaptotagmin 1, which causes an ~2-fold decrease in the $Ca^{2+}$ affinity of synaptotagmin 1 and a similar decrease in the $Ca^{2+}$ responsiveness of synapses (Fernandez-Chacon *et al*., 2001), suggesting that the $Ca^{2+}$ affinity of vesicular synaptotagmins controls synaptic responses. In view of the potential importance of differences in $Ca^{2+}$ affinity, we sought to confirm the measured apparent $Ca^{2+}$ affinities of synaptotagmins with an independent assay. For this purpose we incubated soluble $C_2$A-domain–GST fusion proteins with liposomes at different $Ca^{2+}$ concentrations, isolated the liposomes by centrifugation, and determined the amount of bound synaptotagmin $C_2$A-domains by Coomassie Blue staining of SDS gels. This assay, referred to as the liposome centrifugation assay, utilizes $C_2$A-domains in solution and thus avoids possible immobilization artifacts of the standard resin-based assay. Figure 2 shows that the liposome centrifugation assay produced results similar to the resin-based assay, revealing an ~2-fold higher $Ca^{2+}$ affinity of synaptotagmin 1 than of synaptotagmin 2, and an ~5- to 10-fold higher affinity of the plasma membrane synaptotagmins than of vesicular synaptotagmins. A major difference between the two assays was that in the resin-based assay, a bell-shaped $Ca^{2+}$ concentration dependence was observed for synaptotagmins 3 and 7 (Figure 1), whereas the liposome centrifugation assay exhibited no decrease in phospholipid binding at higher $Ca^{2+}$ concentrations (Figure 2). This difference may reflect the more stringent washes used in the resin-based assay, which would also explain the inability of the resin-based assay to detect $Ca^{2+}$ binding to the $C_2$B-domain (see below; Fernandez *et al*., 2001). The concurrence of the apparent $Ca^{2+}$ affinities determined with the two assays confirms that the $Ca^{2+}$ affinity is not dependent on whether the $C_2$A-domains are immobilized or in solution.

### *Synaptotagmin $C_2$B-domains exhibit similar differences in $Ca^{2+}$ affinity*

Synaptotagmins have two $Ca^{2+}$ binding domains: the $C_2$A- and $C_2$B-domains. Initially the $C_2$B-domains were thought to be unable to bind to phospholipids as a function of $Ca^{2+}$ because the resin-based assay did not detect such binding (reviewed in Südhof and Rizo, 1996). Recently, however, less stringent assays revealed that the $C_2$B-domain of synaptotagmin 1 specifically binds to phospholipids in a $Ca^{2+}$-dependent manner with an apparent $Ca^{2+}$ affinity that resembles that of the $C_2$A-domain (Fernandez *et al*., 2001). The $C_2$B-domain probably forms a less tight $Ca^{2+}$–phospholipid complex than the $C_2$A-domain because the $C_2$B-domain contains only two $Ca^{2+}$ binding sites as opposed to the three $Ca^{2+}$ binding sites of the $C_2$A-domain. To test whether the $C_2$B-domains of synaptotagmins 3 and 7 also bind phospholipids in response to $Ca^{2+}$ and whether the various $C_2$B-domains exhibit similar differences in $Ca^{2+}$ affinity as the $C_2$A-domains, we examined the

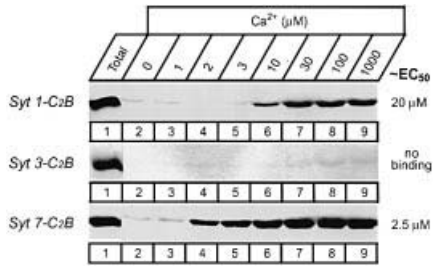


**Fig. 3.** Phospholipid binding by the $C_2B$-domains of synaptotagmins 1, 3 and 7 studied by co-sedimentation with liposomes. Purified GST–$C_2B$-domain fusion proteins were incubated in solution with liposomes composed of 25% PS/75% PC in the presence of the indicated concentrations of free $Ca^{2+}$ and binding was measured as described in Figure 2. Data shown are from a single representative experiment repeated multiple times; numbers on the right indicate approximate $Ca^{2+}$ concentrations required for half-maximal binding as estimated from multiple experiments.

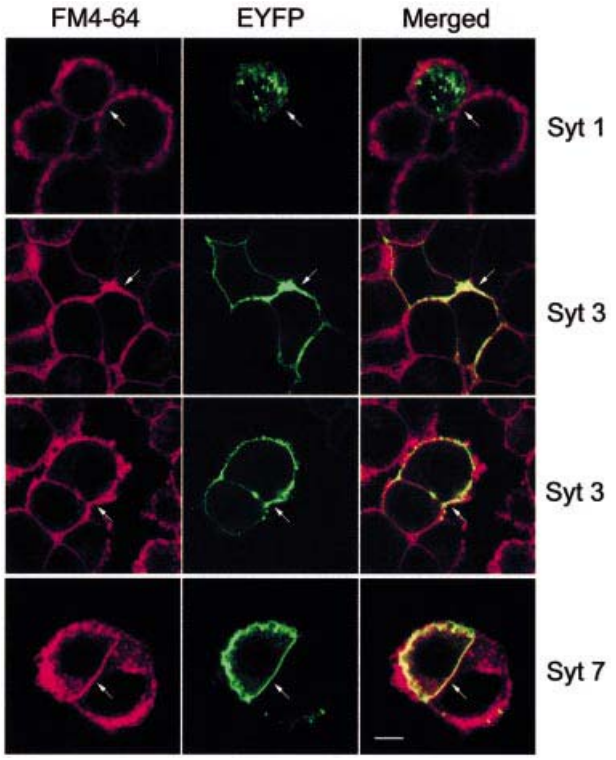


**Fig. 4.** Localization of synaptotagmins 1, 3 and 7 in PC12 cells analyzed with transfected EYFP fusion proteins. PC12 cells were transfected with expression vectors encoding synaptotagmins 1, 3 or 7 as indicated; all synaptotagmins are expressed as C-terminal fusion proteins with EYFP. Unfixed PC12 cells were incubated with the fluorescent dye FM4-64 to stain the plasma membrane and viewed in a confocal microscope. Synaptotagmin 3 is shown in two examples to document reproducibility. Transfected cells are highlighted by a white arrow. The scale bar (2 μm) applies to all panels.

$C_2B$-domains using the liposome centrifugation assay (Figure 2). Figure 3 shows that the synaptotagmin 1 and 7 $C_2B$-domains, but not the synaptotagmin 3 $C_2B$-domain, bind phospholipids as a function of $Ca^{2+}$. The apparent $Ca^{2+}$ affinity of the synaptotagmin 7 $C_2B$-domain was ~10-fold higher than that of the synaptotagmin 1 $C_2B$-domain (Figure 3), demonstrating that the $C_2A$- and $C_2B$-domains of these synaptotagmins exhibit the same difference in $Ca^{2+}$ affinity. The lack of $Ca^{2+}$-dependent phospholipid binding by the synaptotagmin 3 $C_2B$-domain, as judged by this assay, is somewhat surprising considering its sequence similarity to other $C_2B$-domains. It may be due either to a lack of $Ca^{2+}$ binding by this domain (as indicated by the crystal structure; Sutton *et al*., 1999) or to a selective inability to bind phospholipids as a function of $Ca^{2+}$.

### *The relative $Ca^{2+}$ affinities of synaptotagmin $C_2A$-domains are independent of $Ca^{2+}$ buffers*

The apparent $Ca^{2+}$ affinities for synaptotagmin $C_2A$-domains determined above and in previous studies (Fernandez-Chacon *et al*., 2001) were determined with the use of $Ca^{2+}$/EGTA buffers, which may introduce systematic errors. Furthermore, although $Ca^{2+}$ binding assays and secretion measurements in permeabilized PC12 cells both employ $Ca^{2+}$/EGTA buffers, these buffers have distinct compositions (Gerber *et al*., 2001; Sugita *et al*., 2001). To better relate $Ca^{2+}$-dependent phospholipid binding to $Ca^{2+}$-induced secretion in PC12 cells and to evaluate the accuracy of the $Ca^{2+}$/EGTA buffers, we directly compared three different $Ca^{2+}$ buffers: the traditional NaCl-based $Ca^{2+}$/EGTA buffer (Gerber *et al*., 2001); the K-glutamate $Ca^{2+}$/EGTA buffer containing 2 mM $Mg^{2+}$, 2 mM ATP and 0.1% bovine serum albumin (BSA), which is used for permeabilized PC12 cell experiments (Sugita *et al*., 2001); and a NaCl-based buffer, which contained only $Ca^{2+}$ but no EGTA (see Materials and methods). All three buffer systems generally gave similar results, suggesting that the $Ca^{2+}$/EGTA buffers are reliable (data not shown). The only major difference noted was that the synaptotagmin 1 $C_2A$-domain displayed a lower apparent $Ca^{2+}$ affinity in the K-glutamate $Ca^{2+}$/EGTA buffer than in the traditional $Ca^{2+}$/EGTA buffer or the $Ca^{2+}$-only buffer, probably because the ATP in the K-glutamate buffer inhibits phospholipid binding to the $C_2A$-domain of synaptotagmin 1 but not to that of the other synaptotagmins (data not shown).

### *Synaptotagmins in PC12 cells*

As a first approach to testing the functional consequences of the different $Ca^{2+}$ affinities of synaptotagmins, we employed neuroendocrine PC12 cells as a model system. These cells were chosen because they have been productively used in studying $Ca^{2+}$-triggered exocytosis (Ahnert-Hilger *et al*., 1987; Walent *et al*., 1992; McFerran *et al*., 1998; Avery *et al*., 2000) and are known to express at least synaptotagmins 1, 3 and 7 (Shoji-Kasai *et al*., 1992; Mizuta *et al*., 1994; Sugita *et al*., 2001). Previous studies have shown that synaptotagmin 1 is present on secretory vesicles in neuroendocrine cells, while synaptotagmin 7 is localized to plasma membranes (Perin *et al*., 1991; Sugita *et al*., 2001). The localization of synaptotagmin 3, however, is unclear. Some studies in pancreatic β-cells (the only endocrine cell where it has been studied) reported synaptotagmin 3 on secretory vesicles (Mizuta *et al*., 1997; Brown *et al*., 2000; Gao *et al*., 2000), although a more recent study detected it on the plasma membrane (Gut *et al*., 2001), similar to its localization in brain (Butz *et al*., 1999). To address this discrepancy with an independent approach, we examined in PC12 cells the localization of transfected synaptotagmin 3–EYFP fusion protein in

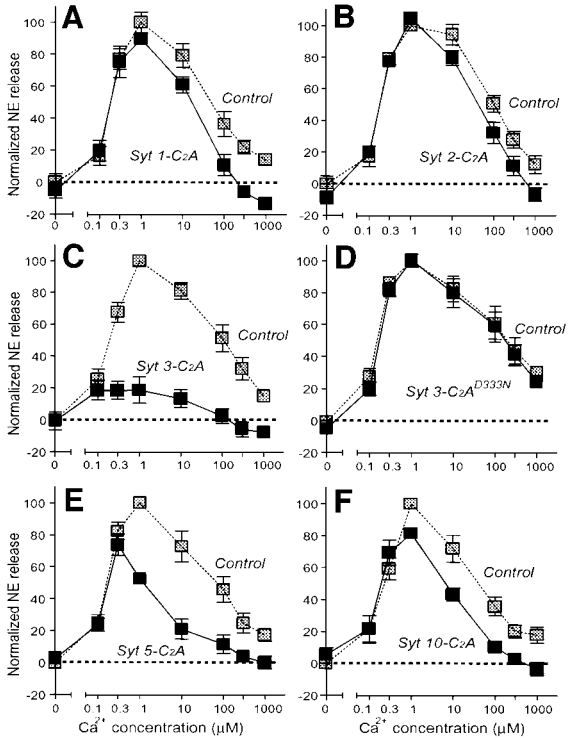


**Fig. 5.** Inhibition of $Ca^{2+}$-triggered exocytosis in permeabilized PC12 cells by the $C_2A$-domains of synaptotagmins 1, 2, 3, 5 and 10. PC12 cells were loaded with $^3H$-labeled norepinephrine, permeabilized and pre-incubated with the indicated purified GST fusion proteins of wild-type synaptotagmin $C_2A$-domains (**A–C**, **E** and **F**) or mutant synaptotagmin 3 $C_2A$-domain containing a single amino acid substitution in the $Ca^{2+}$ binding site (D333N) (**D**). Exocytosis was triggered by addition of $Ca^{2+}$ clamped at the concentrations shown with $Ca^{2+}$/EGTA buffers. Norepinephrine release was normalized to 100% for the maximal release observed under control conditions run in parallel for each experiment with GST (gray symbols). Synaptotagmin $C_2A$-domains and GST were added at 6 and 9 μM, respectively. Data are means ± SEMs from three experiments performed in duplicate and repeated multiple times. Note that the control traces in each graph (amount of secretion in the same assays observed with addition of 9 μM GST alone) differ slightly between graphs because each assay was performed with its own separate controls.

comparison with other synaptotagmins. This approach was employed because the low abundance of synaptotagmin 3 made detection of the endogenous protein difficult, and because the direct fluorescence of EYFP avoids possible artifacts due to indirect immunofluorescence procedures. Figure 4 shows that synaptotagmin 3 is quantitatively deposited into the plasma membrane, which is labeled with the fluorescent dye FM4-64. The localization of synaptotagmin 3 is identical to that of synaptotagmin 7–EYFP, whereas synaptotagmin 1–EYFP is exclusively present in intracellular vesicles (Figure 4). These findings suggest that synaptotagmins 3 and 7 are general plasma membrane proteins in neurons and endocrine cells.

### *Inhibition of $Ca^{2+}$-dependent norepinephrine secretion from permeabilized PC12 cells by synaptotagmin C2-domains*

We loaded PC12 cells with radioactive norepinephrine, permeabilized the cells by freeze–thawing and triggered exocytosis by addition of $Ca^{2+}$ at increasing concentrations. In the absence of protein additions or in the presence of only GST, we observed a bell-shaped $Ca^{2+}$ response curve for exocytosis from the permeabilized PC12 cells (control in Figure 5A). This curve was similar to the $Ca^{2+}$-concentration dependence of phospholipid binding by synaptotagmins 3 and 7 as measured by the resin assay (Figure 1; Sugita *et al.*, 2001). Addition of the synaptotagmin 1 GST–$C_2A$-domain fusion protein to the PC12 cells caused a small but significant inhibition of exocytosis at high $Ca^{2+}$ concentrations (Figure 5A). A similar but lower amount of inhibition was also observed for the $C_2A$-domain of synaptotagmin 2 (Figure 5B), consistent with its lower apparent $Ca^{2+}$ affinity (Figures 1 and 2). The $C_2A$-domain of synaptotagmin 3, however, almost abolished $Ca^{2+}$-triggered exocytosis at all $Ca^{2+}$ concentrations (Figure 5C). This inhibition is similar to the effect we previously observed with the $C_2A$-domain of synaptotagmin 7 (Sugita *et al.*, 2001). Inhibition by the $C_2A$-domain of synaptotagmin 3 required $Ca^{2+}$ binding to the $C_2A$-domain because the $Ca^{2+}$ binding site mutant of the $C_2A$-domain (D333N), which is unable to mediate $Ca^{2+}$-dependent phospholipid binding (Figure 1C), had no effect on exocytosis (Figure 5D). Finally, the $C_2A$-domains of synaptotagmins 5 and 10 had an intermediate effect and significantly inhibited $Ca^{2+}$-triggered exocytosis only at higher $Ca^{2+}$ concentrations (Figure 5E and F).

The PC12 cell experiments suggest that the $Ca^{2+}$ sensor responsible for triggering PC12 cell exocytosis is activated by low micromolar $Ca^{2+}$ concentrations, which precisely match the apparent $Ca^{2+}$ affinities of synaptotagmins 3 and 7, and is inhibited by the $C_2A$-domains from these synaptotagmins. The $Ca^{2+}$ binding measurements described above showed that the $C_2B$-domains of synaptotagmins 1 and 7 exhibited similar differences in $Ca^{2+}$ affinities, raising the question of whether this also applies to the PC12 cell inhibitions. To test this, we measured the effects of the synaptotagmin 1, 3 and 7 $C_2B$-domains on PC12 cell exocytosis. Figure 6 shows that the synaptotagmin 1 and 3 $C_2B$-domains had little effect on $Ca^{2+}$-triggered exocytosis, whereas the synaptotagmin 7 $C_2B$-domain effectively inhibited exocytosis at all $Ca^{2+}$ concentrations. These results again agree well with the $Ca^{2+}$-dependent phospholipid binding measurements (Figure 3), indicating that there is an overall correlation for synaptotagmins in their apparent $Ca^{2+}$ affinities as measured by $Ca^{2+}$-dependent phospholipid binding and their effects in PC12 cells.

We had previously found that the synaptotagmin 7 $C_2A$-domain inhibits $Ca^{2+}$-triggered exocytosis at all $Ca^{2+}$ concentrations except very low ones (Sugita *et al.*, 2001), and a similar trend was observed for the synaptotagmin 3 $C_2A$-domain (Figure 5C). Interestingly, the synaptotagmin 7 $C_2B$-domain exhibited the opposite behavior, in that inhibition of exocytosis was most effective at low $Ca^{2+}$ concentrations (Figure 6C), suggesting that exocytosis at very low $Ca^{2+}$ concentrations is primarily mediated by this $C_2$-domain, and is not due to calmodulin. This suggestion is also supported by the finding that $Sr^{2+}$, a divalent cation that is unable to activate calmodulin (Chao *et al.*, 1984) but does bind to synaptotagmins, strongly activates exocytosis in PC12 cells (see below) and that calmodulin antagonists had no effect on exocytosis under our assay conditions (data not shown).

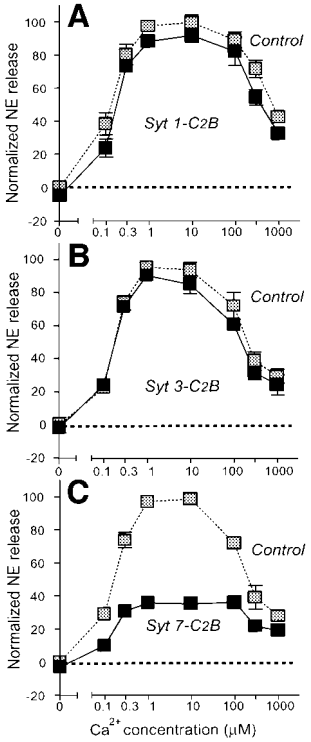


**Fig. 6.** Inhibition of $Ca^{2+}$-triggered exocytosis in permeabilized PC12 cells by the $C_2B$-domains of synaptotagmins 1 (**A**), 3 (**B**) and 7 (**C**). PC12 cells were loaded with $^3H$-labeled norepinephrine, permeabilized, pre-incubated with the indicated purified GST fusion proteins of synaptotagmin $C_2B$-domains or GST alone, and stimulated for exocytosis as described in Figure 5. Data are means ± SEMs from two experiments performed in duplicate.

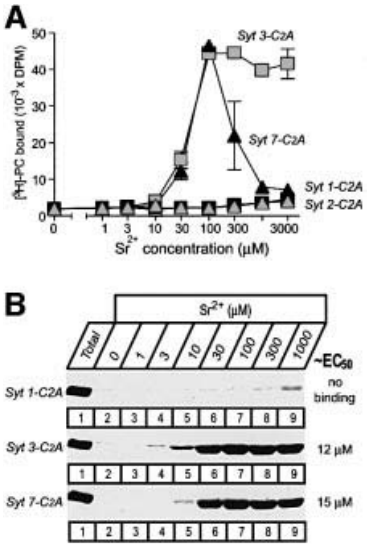


**Fig. 7.** $Sr^{2+}$-dependent phospholipid binding by synaptotagmin $C_2A$-domains measured with the resin-based assay (A) or the liposome centrifugation assay (B). (**A**) Liposome binding to immobilized GST fusion proteins containing the $C_2A$-domains shown was measured as described in Figure 1, except that binding was performed at the indicated concentrations of free $Sr^{2+}$, which were clamped by $Sr^{2+}$/EGTA buffers. Data shown are means ± SEMs from two representative experiments performed in triplicate. (**B**) Liposome binding of GST fusion proteins containing the indicated $C_2A$-domains was measured as described in Figure 2, except that binding was performed at the indicated concentrations of free $Sr^{2+}$ clamped by $Sr^{2+}$/EGTA buffers.

### *$Sr^{2+}$ as a $Ca^{2+}$ mimetic for synaptotagmins and PC12 cell exocytosis*

As an independent approach to test the potential $Ca^{2+}$ sensor function of synaptotagmins in exocytosis, we examined the ability of $Sr^{2+}$ to substitute for $Ca^{2+}$. Differently to previous studies (Li *et al.*, 1995b), the current experiments were performed using $Sr^{2+}$/EGTA buffers to control the free $Sr^{2+}$ concentration more precisely, to eliminate potential interference by contaminating $Ca^{2+}$ and to allow a direct comparison of $Ca^{2+}$ binding and of $Ca^{2+}$ triggered exocytosis in PC12 cells. Figure 7 shows that at all $Sr^{2+}$ concentrations examined, the $C_2A$-domains of vesicular synaptotagmins 1 and 2 did not bind to phospholipids, whereas synaptotagmins 3 and 7 exhibited similar apparent $Sr^{2+}$ affinities in both buffers ($EC_{50} \approx 30$–$50$ μM). The resin-based and the liposome centrifugation assays for measuring the apparent $Sr^{2+}$ affinity gave identical results (Figure 7). The specificity of the reactions was confirmed with the synaptotagmin 3 mutant D333N, which, similarly to $Ca^{2+}$-dependent phospholipid binding (Figure 1C), was unable to support $Sr^{2+}$-dependent phospholipid binding (data not shown).

We next measured the response of permeabilized PC12 cells to increasing $Sr^{2+}$ concentrations and examined the effects of recombinant synaptotagmin $C_2A$-domains on this response. As before, GST alone was employed in all experiments as a negative control analyzed in parallel with the test proteins. Under control conditions, $Sr^{2+}$ was as effective as $Ca^{2+}$ in triggering exocytosis (Figure 8). A bell-shaped $Sr^{2+}$ concentration curve was measured similarly to the $Ca^{2+}$ concentration curve, although much higher $Sr^{2+}$ concentrations (100–300 μM) were required for maximal effect. Similarly to $Ca^{2+}$-triggered exocytosis, the synaptotagmin 1 $C_2A$-domain had only a limited effect on $Sr^{2+}$-triggered exocytosis, with a moderate inhibition at high $Sr^{2+}$ concentrations (Figure 8A). In contrast, the wild-type synaptotagmin 3 $C_2A$-domain completely abolished $Sr^{2+}$-triggered exocytosis (Figure 8B), but the mutant synaptotagmin 3 $C_2A$-domain (which is unable to form $Sr^{2+}$-dependent phospholipid complexes; see Figure 7) had no effect (Figure 8C). The synaptotagmin 7 $C_2A$-domain was even more effective in inhibiting exocytosis than the synaptotagmin 3 $C_2A$-domain, and depressed release below non-stimulated control levels (Figure 8D).

## Discussion

Synaptotagmins constitute a large family of membrane proteins that are preferentially expressed in brain and are composed of an N-terminal transmembrane region, a linker sequence and two C-terminal $C_2$-domains. Synaptotagmin 1, the first synaptotagmin studied, is an abundant synaptic vesicle protein that binds $Ca^{2+}$ and phospholipids via both of its $C_2$-domains and is essential for fast $Ca^{2+}$-triggered neurotransmitter release (reviewed in Südhof and Rizo, 1996). A large body of evidence supports a role for synaptotagmin 1 and its close homolog synaptotagmin 2 as a $Ca^{2+}$ sensor in exocytosis, especially the observation that changes in the $Ca^{2+}$ affinity of synaptotagmin 1 produce a coordinate change in the

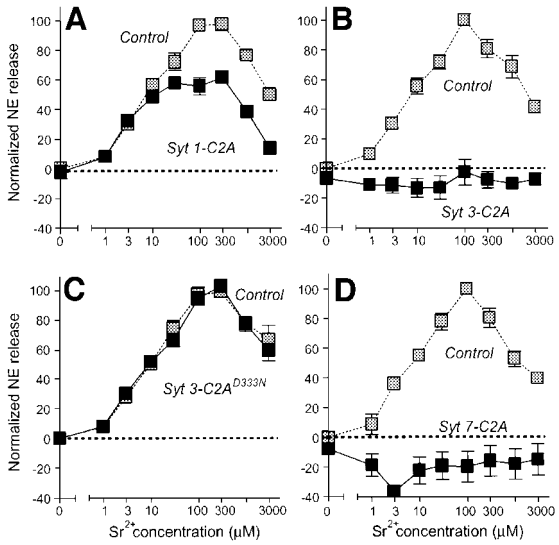


**Fig. 8.** Inhibition of $Sr^{2+}$-triggered exocytosis in permeabilized PC12 cells by the $C_2A$-domains of synaptotagmins 1 (**A**), 3 (**B** and **C**) and 7 (**D**). PC12 cells were loaded with $^3H$-labeled norepinephrine, permeabilized and pre-incubated with the indicated purified GST fusion proteins of synaptotagmin $C_2A$-domains. In the case of the synaptotagmin 3 $C_2A$-domain, a wild-type (B) and a mutant $C_2A$-domain (C) containing a single amino acid substitution in the $Ca^{2+}$ binding site (D333N) were tested. Exocytosis was triggered by addition of the indicated concentrations of $Sr^{2+}$ clamped with $Sr^{2+}$/EGTA buffers, and normalized to 100% for the maximal release observed under control conditions run in parallel (gray symbols). Data are means ± SEMs from two experiments performed in duplicate.

$Ca^{2+}$ affinity of neurotransmitter release (Fernandez-Chacon *et al*., 2001). Nevertheless, multiple observations indicate that neurotransmitter release is not triggered by synaptotagmins 1 and 2 alone. These include the findings that some $Ca^{2+}$-triggered release remains in synapses lacking synaptotagmins 1 and 2 (Geppert *et al*., 1994), chromaffin cells deficient in synaptotagmin 1 exhibit a selective impairment in the minor fast component of $Ca^{2+}$-triggered exocytosis (Voets *et al*., 2001) and PC12 cells without synaptotagmins 1 or 2 are capable of relatively normal $Ca^{2+}$-stimulated exocytosis (Shoji-Kasai *et al*., 1992). Although other synaptotagmins, such as synaptotagmins 3 and 7, are present at synapses and in neuroendocrine cells in addition to synaptotagmin 1, these synaptotagmins are localized to the plasma membrane instead of to synaptic vesicles (Butz *et al*., 1999; Sugita *et al*., 2001; Figure 4). At synapses, the plasma membrane synaptotagmins are clearly not redundant with synaptotagmin 1 because the synaptotagmin 1 knockout has a strong synaptic phenotype, but the other synaptotagmins may mediate $Ca^{2+}$-triggered exocytosis in endocrine cells that do not require synaptotagmin 1 (Shoji-Kasai *et al*., 1992; Sugita *et al*., 2001). Viewed together, these findings raised the possibility that different synaptotagmins are specialized for distinct $Ca^{2+}$-regulated functions in exocytosis that correlate with their subcellular localizations. We have tested this hypothesis by examining the apparent $Ca^{2+}$ affinities of the most abundant synaptotagmins and by relating these affinities to $Ca^{2+}$-triggered exocytosis in neuroendocrine PC12 cells.

Our major finding is that different synaptotagmins exhibit distinct apparent $Ca^{2+}$ affinities (Figures 1–3). Affinities were measured by $Ca^{2+}$-dependent phospholipid binding, which most likely represents the physiological $Ca^{2+}$ binding activity of $C_2$-domains, because phospholipid binding is the best functionally validated general property of $C_2$-domains and because the apparent $Ca^{2+}$ affinity of the $C_2$-domains of synaptotagmins in the presence of phospholipids (<0.05 mM $Ca^{2+}$), but not in their absence (>0.4 mM $Ca^{2+}$), is in the physiological range (Ubach *et al*., 1998; Zhang *et al*., 1998; Fernandez *et al*., 2001; Fernandez-Chacon *et al*., 2001). We have examined both $C_2$-domains of the major synaptotagmins. To rule out potential artifacts created, for example, by the use of $Ca^{2+}$/EGTA buffers or of immobilized GST fusion proteins, we have confirmed the results by independent methods in multiple buffer systems. Our measurements showed that synaptotagmin 2 has an ~2-fold lower $Ca^{2+}$ affinity than synaptotagmin 1, providing the first evidence for a functional divergence between these two differentially distributed isoforms of vesicular synaptotagmins. More importantly, our data demonstrated that synaptotagmins 3 and 7 have an ~5- to 20-fold higher $Ca^{2+}$ affinity than synaptotagmins 1 and 2, suggesting that the plasma membrane synaptotagmins are activated at lower $Ca^{2+}$ concentrations than the vesicular synaptotagmins. Moreover, synaptotagmins 5 and 10 also exhibited relatively high $Ca^{2+}$ affinities, whose significance is difficult to evaluate because the localizations of these synaptotagmins are unknown. It should be noted that the $Ca^{2+}$ affinities described here are not absolute but depend on the composition of the phospholipid membranes and on the ionic strength (Zhang *et al*., 1998; Fernandez-Chacon *et al*., 2001). Our numbers therefore provide only general figures for the relative $Ca^{2+}$ affinities of the various synaptotagmins. The 'true' affinities may vary by a factor of 2–4, depending on the precise lipid composition of the sites of exocytosis, which is unknown. Furthermore, double $C_2$-domain fragments probably have higher apparent $Ca^{2+}$ affinities than individual $C_2$-domains.

Our second major finding is that the apparent $Ca^{2+}$ affinities of synaptotagmin $C_2$-domains correlate with their ability to inhibit $Ca^{2+}$-stimulated exocytosis in PC12 cells (Figures 5 and 6). Among others, we show that both of the currently known synaptic plasma membrane synaptotagmins, synaptotagmins 3 and 7, are also present on plasma membranes in PC12 cells (Figure 4) and that interference with their functions severely impairs exocytosis, suggesting that these two synaptotagmins together serve as plasma membrane $Ca^{2+}$ sensors for exocytosis (Figure 5). The mechanism by which the $C_2$-domains of these synaptotagmins block exocytosis is unknown, but the inhibition is clearly specific, as shown by the differential effects observed with various phospholipid-binding $C_2$-domains (Figure 5) and confirmed in control experiments with $C_2$-domains from another synaptic protein, rabphilin (data not shown). The functional analysis of $C_2$-domains was extended to PC12 cells where exocytosis was stimulated with $Sr^{2+}$ instead of $Ca^{2+}$: $Sr^{2+}$-triggered exocytosis was also abolished by the $C_2A$-domains of synaptotagmins 3 and 7, but not by synaptotagmin 1 (Figure 8). Based on these observations, we propose an overall model whereby two classes of

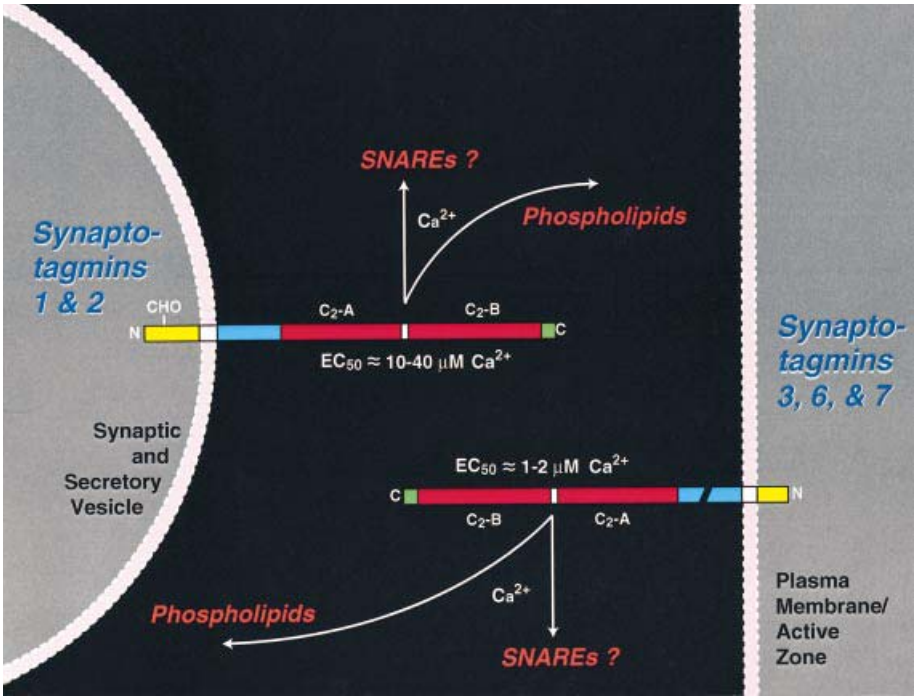


**Fig. 9.** Diagram of the symmetrical localizations, but asymmetrical $Ca^{2+}$ affinities, of synaptotagmins on secretory vesicles and plasma membranes. The structures of synaptotagmins are schematically indicated with extracytoplasmic sequences (yellow; note that vesicular synaptotagmins are glycosylated, as indicated by 'CHO'), transmembrane regions (white), linker sequences (blue; break in the plasma membrane synaptotagmins indicates alternative splicing in synaptotagmin 7), $C_2$-domains (red) and C-terminal sequences (green). The $C_2$-domains of synaptotagmins are thought to bind to phospholipids as a function of $Ca^{2+}$ and possibly also to SNARE proteins. The apparent affinities of the $C_2$-domains in the presence of negatively charged phospholipids are decribed next to the $C_2$-domains. $Ca^{2+}$ affinities shown apply to single $C_2$-domains in the presence of negatively charged phospholipids. Because of cooperativity between $C_2$-domains, the double $C_2$-domain fragments have an ~3-fold higher $Ca^{2+}$ affinity.

synaptotagmins, vesicular synaptotagmins 1 and 2 and plasma membrane synaptotagmins 3 and 7, execute distinct functions in exocytosis (Figure 9). The central tenet of this working model is that the two classes of synaptotagmins cooperate in exocytosis, with the high-affinity plasma membrane synaptotagmins mediating $Ca^{2+}$-triggered exocytosis in neuroendocrine cells and the slow component of synaptic vesicle exocytosis, whereas the low-affinity vesicular synaptotagmins are largely dispensable for slow neuroendocrine exocytosis but are required for fast synaptic vesicle exocytosis. It has been suggested that calmodulin may be a major $Ca^{2+}$ sensor for exocytosis (Chen *et al*., 1999; Quetglas *et al*., 2000) instead of modulating release as originally proposed (Chamberlain *et al*., 1995; Kibble and Burgoyne, 1996). Our data demonstrating that $Sr^{2+}$ is fully capable of triggering release rule out an essential role for calmodulin as a $Ca^{2+}$ trigger for exocytosis in PC12 cells.

Our findings are at odds with some of the observations on synaptotagmins reported in the literature. In pancreatic β-cells, synaptotagmin 3 was found on secretory vesicles instead of the plasma membrane (Mizuta *et al*., 1997; Brown *et al*., 2000; Gao *et al*., 2000). However, these studies used antibodies that recognized either no synaptotagmin 3 protein in brain, or a protein of different size. Since synaptotagmin 3 is primarily expressed in brain and not subject to alternative splicing (Li *et al*., 1995a; Südhof, 2002), this result raises the possibility that the antibodies did not react with synaptotagmin 3 with high affinity. Indeed, this possibility was confirmed by Gut *et al*. (2001), who showed with affinity-purified antibodies that synaptotagmin 3 is on the plasma membrane in pancreatic β-cells. Another discrepancy with published data (Desai *et al*., 2000) is the fact that we observe no significant inhibition of exocytosis by the $C_2$A- or $C_2$B-domain of synaptotagmin 1 at $Ca^{2+}$ concentrations <10 μM, but do see inhibition at concentrations >0.1 mM $Ca^{2+}$. However, our results demonstrate that the overall $Ca^{2+}$ affinity of synaptotagmin 1, both of the $C_2$A- and the $C_2$B-domain, is much lower than the $Ca^{2+}$ response curve of exocytosis (compare Figure 1A with 6A). These data are in perfect agreement with previous analyses of cells lacking synaptotagmin 1, showing that this synaptotagmin is not required for the vast majority of neuroendocrine exocytosis (Shoji-Kasai *et al*., 1992; Voets *et al*., 2001).

The finding that different synaptotagmins exhibit distinct apparent $Ca^{2+}$ affinities and subcellular localizations may have significant functional implications. The difference in $Ca^{2+}$ affinities between synaptotagmins 1 and 2 suggests that these highly homologous synaptotagmins, which are differentially distributed but otherwise similar in subcellular localization (Geppert *et al*., 1991; Ullrich *et al*., 1994), could contribute to the $Ca^{2+}$ responsiveness of a synapse. Our data thus predict that synapses expressing synaptotagmin 2 require higher $Ca^{2+}$ concentrations for release than those expressing synaptotagmin 1. Furthermore, vesicular and plasma membrane synaptotagmins with distinct $Ca^{2+}$ affinities

appear to be co-expressed on opposing membranes in synapses. Since $Ca^{2+}$ influx during an action potential causes a rapid increase, but slower decline in the synaptic $Ca^{2+}$ concentration, plasma membrane and vesicular synaptotagmins are presumably activated in parallel. However, as the $Ca^{2+}$ concentration declines, the plasma membrane synaptotagmins may remain active, whereas vesicular synaptotagmins are shut off. This would suggest that the remaining slow component in synaptotagmin 1 knockout mice is at least partly mediated by plasma membrane synaptotagmins. Moreover, if the relative abundance of various synaptotagmins differed between synapses, this could potentially explain the differences in release probabilities between nerve terminals. For example, layer 2/3 pyramidal cells in the cortex form synapses with a high probability of release with bitufted interneurons, and synapses with a low probability of release with multipolar interneurons (Rozov *et al*., 2001). Other examples are the parallel fiber and the climbing fiber synapses in cerebellum, which have similar sizes and numbers of docked vesicles, but which exhibit an up to 10-fold difference in release probability (Xu-Friedman *et al*., 2001). One factor that might contribute to these differences is the mix of synaptotagmins that is expressed at these synapses. Again, future experiments will have to test whether such differences are, at least in part, caused by variations in synaptotagmins.

## Materials and methods

### *Expression and purification of recombinant proteins*

All recombinant proteins were expressed as bacterial GST fusion proteins in pGEX-KG (Guan and Dixon, 1991). Most vectors have been described previously (Sugita *et al*., 2001) except for the synaptotagmin 3 plasmids, which were constructed by PCR using standard methods (Sambrook *et al*., 1989). GST fusion proteins were purified essentially as described (Guan and Dixon, 1991; Sugita and Südhof, 2000; Sugita *et al*., 2001) and $C_2B$-domain proteins were additionally treated to remove the bacterial contaminants that stick to these domains (Ubach *et al*., 2001). For this purpose, extracted proteins were incubated with 1500 U/l benzonase (Novagen Inc.) for 1 h at room temperature before binding to glutathione–agarose. The agarose was packed into a polypropylene column with a paper disc column (Quick-Sep, Isolab) and additionally washed five times with 5 ml of phosphate-buffered saline (PBS), 1 M NaCl, 1% Triton X-100, 1 mM EDTA and 0.1 g/l phenylmethylsulfonyl fluoride (PMSF), followed by six washes with 5 ml of PBS, 1 M NaCl, 1 mM EDTA and 0.1 g/l PMSF.

### *Phospholipid binding assays*

These were performed essentially as described using GST fusion proteins either immobilized on glutathione–agarose in a resin assay (Davletov and Südhof, 1993; Li et al., 1995a,b) or in solution with a centrifugation assay (Fernandez *et al*., 2001) with three different buffers. Buffer A: NaCl-based $Ca^{2+}$/EGTA buffer (50 mM HEPES–NaOH pH 6.8, 0.1 M NaCl, 4 mM $Na_2EGTA$, and total $Ca^{2+}$ or $Sr^{2+}$ to produce the indicated concentrations of free $Ca^{2+}$ or $Sr^{2+}$); buffer B: K-glutamate-based $Ca^{2+}$/EGTA buffer (120 mM K-glutamate, 20 mM K-acetate, 2 mM EGTA, 20 mM HEPES–NaOH pH 7.2, and total $Ca^{2+}$ or $Sr^{2+}$ to produce the indicated concentrations of free $Ca^{2+}$ or $Sr^{2+}$); buffer C: NaCl-based $Ca^{2+}$-only buffer [50 mM HEPES–NaOH pH 6.8; 0.1 M NaCl passed through AG MP-50 resin (Bio-Rad) to eliminate $Ca^{2+}$ contamination after resin ion conversion to $Na^+$ from $H^+$, with $Ca^{2+}$ or $Sr^{2+}$ added directly to the buffer from 1 M stock solution (Fluka Chemical Corp., NY) to achieve the indicated total $Ca^{2+}$ or $Sr^{2+}$ concentration]. For composition of $Ca^{2+}$/EGTA and $Sr^{2+}$/EGTA buffers, the respective amounts of total $Ca^{2+}$ or $Sr^{2+}$ needed to achieve a defined free $Ca^{2+}$ or $Sr^{2+}$ concentration were calculated with EqCal software (Biosoft, Ferguson, MI) using buffer constants given by the program or published by Martell and Smith (1974). All buffers were prepared in high-resistance MilliQ water using a 1 M $Ca^{2+}$ standard solution (Fluka Chemical Corp.).

*Resin assay*. PS and PC (1.75 mg total; Avanti Polar Lipids, AL) were dissolved in chloroform, mixed in a 1:3 weight ratio including <0.01% $^3$H-PC (Amersham Pharmacia Biotech, NJ) and dried under a stream of nitrogen. Dried lipids were resuspended in $Ca^{2+}$-free buffer A or B (10 ml) by vigorous vortexing for 1 min, sonicated for 5 min in a waterbath sonicator (model G112PIG; Laboratory Supply Co. Inc., NJ; output: 80 kc, 80 W) and centrifuged (15 min at ~5000 *g*) to remove aggregates. Binding assays contained ~25 µg of recombinant protein, with 1 g protein/l wet glutathione beads. Beads were equilibrated in 0.1 ml of the respective binding buffers (buffer A or B with 8.75 µg of phospholipids and 0.025 µCi of $^3$H-PC). Binding reactions were incubated for 10 min at 30°C with vigorous shaking, briefly centrifuged, washed three times with 0.8 ml of the respective binding buffers and phospholipid binding quantified by scintillation counting of the beads (Beckman LS6000SC; Beckman Instruments Inc., CA).

*Centrifugation assay*. Phospholipids (PS/PC = 25/75, w/w) in chloroform were dried as a thin layer under a stream of nitrogen. Buffers A, B or C containing 0.5 M sucrose were added to the dried phospholipid layer, vortexed for 20 min and sonicated for 5 min in a bath sonicator (model G112SP1G; Laboratory Supply Co. Inc.). After liposome formation, 4 vols of each buffer without sucrose were added and centrifuged to separate heavy liposome from free phospholipids (100 000 *g* for 30 min). Heavy liposomes were washed once and re-pelleted (13 000 r.p.m. for 10 min). Liposomes were resuspended in each buffer with various concentrations of free $Ca^{2+}$ and used within 1 h. Purified soluble recombinant GST–synaptotagmin $C_2$-domains (6 µg) and liposomes (100 µg of phospholipids; total volume = 1 ml) were incubated for 10 min on an Eppendorf thermal mixer at 30°C and 1400 r.p.m., liposomes were re-isolated by centrifugation (20 800 *g* for 10 min) and washed three times with 0.5 ml of the corresponding buffers. Chloroform:methanol (1:2, v/v) solution was added into the pelleted liposomes to denature protein and dissolve phospholipids. After centrifugation (20 800 *g* for 15 min), the protein precipitate was resuspended in 30 µl of 2× SDS sample buffer and analyzed by SDS–PAGE and Coomassie Blue staining.

### *Permeabilized PC12 cell secretion assay*

Secretion assays were conducted using freeze–thaw permeabilization of PC12 cells (Klenchin *et al*., 1998; Sugita *et al*., 2001). PC12 cells were maintained in 75 cm$^2$ flasks (uncoated) in RPMI1640 with 5% fetal bovine serum (heat inactivated), 10% horse serum (heat inactivated), penicillin (50 U/ml) and streptomycin (50 U/ml) at 37°C in 5% $CO_2$. For each assay, PC12 cells were plated in 10 cm$^2$ dishes (Costar) at 40–50% confluency. Two to three days after plating (70% confluency), PC12 cells fresh medium (total 10 ml) containing 4 µl of [$^3$H]norepinephrine (NEN; 1 µCi/µl in stock) and 0.5 mM ascorbic acid was added. After 24 h, cells were washed with physiological saline solution (145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM HEPES–NaOH pH 7.4), detached from the wells by pipetting a stream of $Ca^{2+}$-free ice-cold buffer B, washed twice with 6 ml of ice-cold buffer B and resuspended in 6 ml of buffer B in a 15 ml cone tube. For permeabilization, cells were frozen overnight at –80°C, thawed by warming the tube at room temperature, 10 mM EGTA was added and the thawed cells were left on ice for 1–5 h to allow efficient extraction of soluble proteins. Resulting cell ghosts were washed three times with 3 ml of ice-cold $Ca^{2+}$-free buffer B containing 1 g/l BSA, with centrifugations at 1000 *g* for 5 min. About 18–36 secretion reactions were conducted with each preparation with standard reaction mixes containing in 1.5 ml microcentrifuge tubes (total volume 0.1 ml): washed cell ghosts, 2 mM ATP, 2 mM $MgCl_2$, 10 µl rat brain cytosol (10 mg/ml) in buffer B with various concentrations of $CaCl_2$ and recombinant proteins. Reactions were incubated for 30 min at 30°C, terminated by chilling to 0°C and samples were centrifuged at 4°C for 3 min at 20 800 *g*. Supernatants and the pellets solubilized in 1% Triton X-100 were analyzed by liquid scintillation counting.

### *PC12 cell transfections*

Vectors encoding EYFP fusion proteins were constructed in a CMV-based expression plasmid using standard procedures (Sugita *et al*., 2001). For transfection, PC12 cells were plated on polylysine-coated coverslips in RPMI medium supplemented with 10% fetal bovine serum and 5% horse serum. After 1 day in culture, cells were transfected with appropriate vectors using Tfx-50 (Promega). Forty-eight to 72 h after transfection, culture medium was replaced with 0.2 ml of minimal essential medium containing 10 µM FM4-64 (Molecular Probes Inc.) and cells were incubated at 37°C for 5–10 min. FM4-64-labeled coverslips

were mounted on slides and imaged using a Bio-Rad MRC-1024 confocal microscope with a 100× (NA1.3) objective.

### *Miscellaneous procedures*

SDS–PAGE and immunoblotting were performed using standard procedures (Laemmli, 1970; Johnston *et al*., 1989). Immunoblots were developed by enhanced chemiluminescence (Amersham).

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